

Immunotherapy with MVA-BN[®]-HER2 induces HER-2-specific Th1-immunity and alters the intratumoral balance of effector and regulatory T cells

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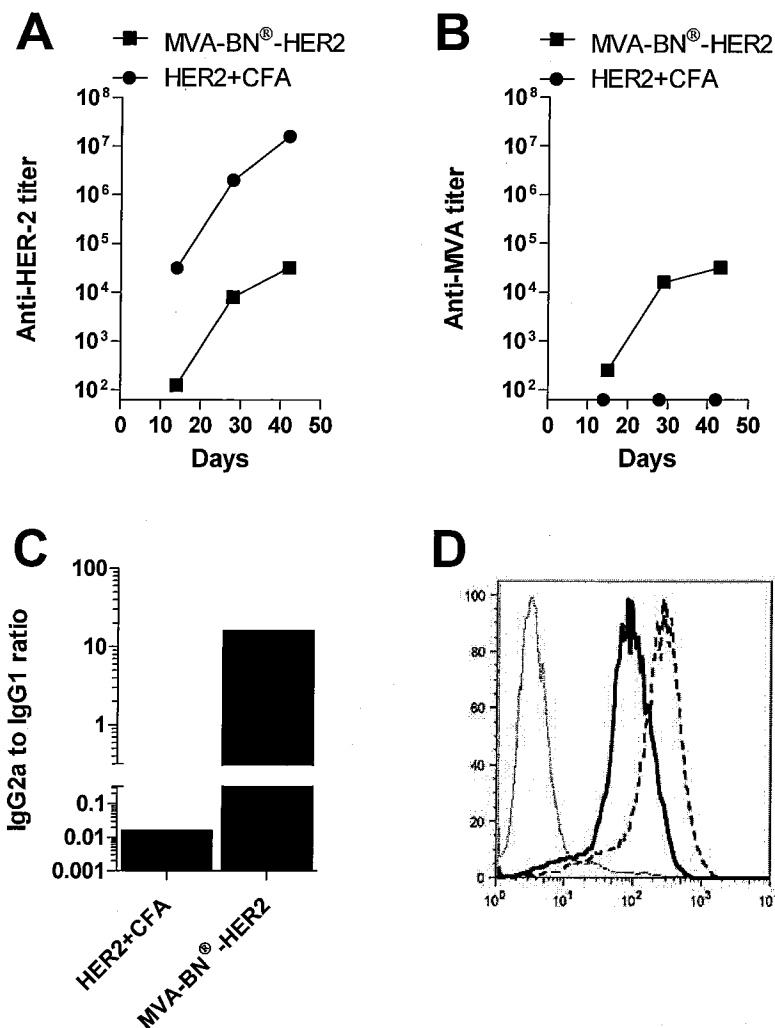
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Supplementary Fig. 1 MVA-BN®-HER2 induces potent Th1-dominated anti-HER-2 antibody responses.

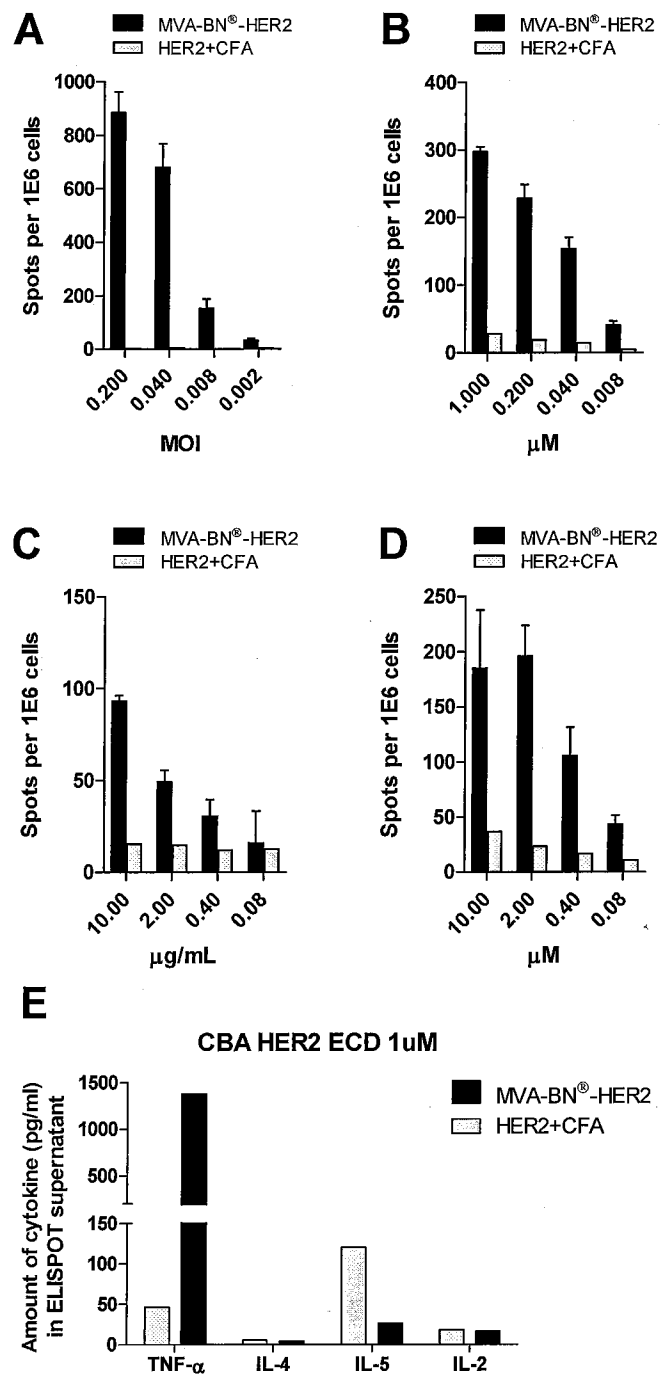
BALB/c mice were treated subcutaneously (s.c.) on days 1, 15, and 29 (q2wks x 3) with 5E7 TCID₅₀ of MVA-BN®-HER2, or 5µg of HER2 protein formulated in CFA, followed by two booster immunizations of 5µg HER2 protein formulated in Incomplete Freund's Adjuvant. Negative controls received Tris Buffered Saline (TBS). Serum was collected from all animals on days 14, 28, and 42, and HER-2 and MVA-specific antibody titers were evaluated by ELISA. **A** Anti-HER-2 antibody titers and **B** anti-MVA antibody titers in pooled sera from 5 mice per group treated either with MVA-BN®-HER2 (squares) or HER2+CFA (circles). **C** IgG2a / IgG1 isotype ratio of anti-HER-2 antibody titers **D** Antibody binding to human HER-2 expressed on CT26 tumor cells (serum at 1:100 dilution from MVA-BN®-HER2-treated animals (black solid line), HER2+CFA-treated animals (dotted line) or TBS-treated animals (grey line)).

Treatment with MVA-BN[®]-HER2 induced HER-2- and MVA-specific antibody responses that increased with multiple administrations reaching a titer of 32,000 on day 42 for both antigens (Supplemental Figure 1A and 1B). Immunization with HER2+CFA induced higher titers than treatment with MVA-BN[®]-HER2 reaching a titer of 16,000,000 by day 42. Further analysis showed that both the kinetics and the overall magnitude of anti-HER-2 and anti-MVA titers are dose-dependent in animals treated with MVA-BN[®]-HER2 (data not shown).

To characterize putative adjuvant activities of the MVA-BN[®] vector on immune responses to the transgene, the ratio of anti-HER-2 IgG2a versus IgG1 antibody subtypes produced upon treatment with MVA-BN[®]-HER2 or HER2+CFA was determined. Immunization with MVA-BN[®]-HER2 induced a strongly Th1-dominated humoral response with an IgG2a/IgG1 ratio of 16 (titer of 4,000 for IgG1 and 64,000 for IgG2a, Supplemental Fig. 1C). Conversely, immunization with HER2+CFA induced a strikingly Th2-dominated antibody response with an antibody ratio of 0.016 (8,000,000 for IgG1 and 128,000 for IgG2a).

To address whether the anti-HER-2 antibodies induced by MVA-BN[®]-HER2 could bind to native HER-2 expressed on tumor cells, CT26-HER-2 cells were incubated with serum from mice treated with TBS, 5E7 TCID₅₀ MVA-BN[®]-HER2 or HER2+CFA. Sera of mice treated with either MVA-BN[®]-HER2 or HER2+CFA contained antibodies that bind cells expressing human HER-2 (Supplemental Fig. 1D).

Together, these results demonstrate that the MVA-BN[®] vector exerts potent Th1-biased adjuvant function towards the co-expressed HER-2-transgene resulting in the induction of strong, Th1-dominated, HER-2-specific antibody responses in mice treated with MVA-BN[®]-HER2. Treatment with HER2+CFA in contrast, induces strong Th2-dominated antibody responses.

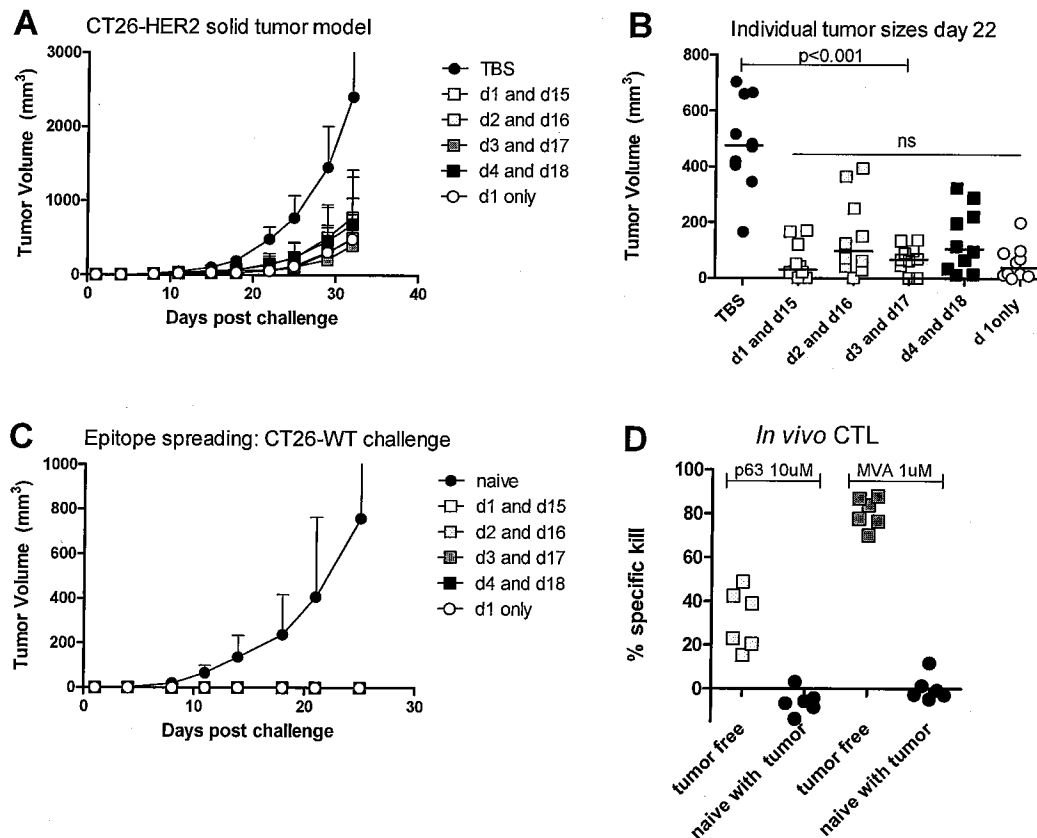


Supplementary Fig. 2 MVA- and HER-2-specific T cell responses in mice treated with MVA-BN®-HER2 or HER2+CFA.

Mice (3 mice/group) were treated on days 1, 15, 29, and 43 (q2wks x 4) with either MVA-BN[®]-HER or HER2+CFA. Seven days after the last treatment splenocytes from each treatment group were pooled and analyzed by IFN γ -ELISpot after restimulation with either: **A** MVA-BN[®], **B** HER-2 ECD overlapping peptide library (166 overlapping 15mers), **C** HER-2 ECD protein, or **D** K^d-binding HER-2 peptide p63. **E** Cytokine profile in supernatants of wells restimulated with 1 μ M HER2 ECD OPL (panel B). For this analysis the "Mouse Th1/Th2 Cytokine kit" (a Cytometric Bead Array from BD, CAT.#551287) was used.

Strong MVA-specific T cell responses were detected in splenocytes of mice treated with MVA-BN[®]-HER2 reaching an average of 880 spots per million splenocytes (MOI 0.2). Under the same conditions only 2-4 IFN- γ spots per million cells were detected in HER2+CFA immunized mice (Supplemental Figure 2A) demonstrating the high specificity of this assay. More importantly, treatment with MVA-BN[®]-HER2 induced 5 to 10-fold higher frequencies of HER-2 specific T cells than HER2+CFA treatment, as seen by restimulation with 1 μ M of a HER-2 overlapping peptide library (HER-2 OPL) (297 spots versus 28, Fig. 2B) or with HER2 protein (Supplemental Figure. 2C). While the addition of HER2 protein or HER-2 OPL could potentially restimulate both CD4 and CD8 T cells, previous depletion experiments have shown that they only stimulate CD4 T cells to produce IFN- γ under the given ELISpot conditions (data not shown). Robust CD8-specific T cell responses were seen in MVA-BN[®]-HER2-treated mice upon restimulation with p63, a K^d-restricted HER-2 peptide, resulting in 5-fold higher T cell responses than observed after treatment with HER2+CFA (Supplemental Figure 2D).

Furthermore, large amounts of TNF- α (1378pg/ml) were found in pooled supernatants from splenocytes of MVA-BN[®]-HER2 treated animals that were restimulated with 1 μ M HER2 ECD OPL, while only marginal amounts of this cytokine were found under the same conditions in the splenocyte supernatants from HER2+CFA treated animals (46pg/ml). The reverse situation was found for IL-5. No difference was seen between treatment groups for IL-2 and IL-4 levels were below the detection limit. Together, these results demonstrate that MVA-BN[®]-HER2 induces potent Th1-dominated HER-2-specific cellular immune responses in mice treated with MVA-BN[®]-HER2 while treatment with HER2+CFA induces Th2-dominated immune responses specific for HER-2.



Supplemental Figure 3: The CT26-HER2 solid tumor model.

A Tumor growth, 1E5 CT26-HER2 cells were injected intradermally (i.d.), tumors were measured twice weekly using calipers. Tumor volume was calculated as $(\text{length} \times \text{width}^2)/2$. On day 32 all TBS-treated animals had to be taken down due to large tumor sizes. **B** Tumor volume of individual mice on day 22. Differences in tumor size at each particular day of tumor measurements were compared by One-Way ANOVA with Bonferroni's multiple comparison post-analysis. Differences compared to the TBS groups remained significant throughout the day the TBS mice had to be taken down (day 32). **C** On day 39 all tumor free mice (10/50 treated with MVA-BN[®]-HER2) were pooled and re-challenged with wild-type CT26 cells (1E5 cells, i.d.). As a control, 10 naïve mice were challenged with CT26 cells also (naïve). **D** 27 days after tumor challenge an *in vivo* CTL assay was performed on 6 tumor-bearing mice from the naïve group, 6 tumor-free mice of the previously MVA-BN[®]-HER2 treated animals that rejected both CT26-HER-2 and CT26-wt tumors, and 2 totally naïve mice to define background. Target cells (splenocytes) were labeled with either a 1μM mixture of 2 MVA epitopes (F2L and E3L) or with 10μM of p63 peptide. This assay was done about 4 weeks after the CT26-wt tumors were rejected, and 2 months since the last MVA-BN[®]-HER2 treatment, so the killing is primarily from a memory, and not effector phase, response.

In this experiment, 10 female BALB/c mice per group were implanted intradermally (i.d.) with 1E5 CT26-HER2 cells (the same cell line as used in the experimental pulmonary metastasis model)

and treatment was started on days indicated in the figure legend. The treatment schedule consisted of 2 vaccinations 2 weeks apart (q2wksx2). 1×10^7 TCID₅₀ of MVA-BN[®]-HER2 were given by skin scarification.

Significant anti-tumor efficacy was observed throughout the study with 20% tumor free mice by day 39 (10/50 across treatment groups). No difference was observed between groups in which treatment was initiated on day 1, 2, 3 or 4. Tumor free mice were re-challenged with CT26-wt tumor (which does not express HER-2). All mice completely rejected the challenge demonstrating that epitope-spreading to other tumor antigens had occurred. An *in vivo* CTL assay demonstrated that long-lasting CTL memory had been induced. 70-90% of MVA target cells labeled with a 1 μ M peptide mix of two MVA-dominant CTL epitopes were killed. In the same mice, 20-50% killing of p63 target cells was observed.

Together, these data demonstrate significant anti-tumor activity against i.d. implanted CT26-HER-2 cells that grow as solid tumors. Furthermore, epitope spreading and long-lasting immune memory was observed.